

# Tumor Necrosis Factor- $\alpha$ Contributes to Apoptosis in Hippocampal Neurons during Experimental Group B Streptococcal Meningitis

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To evaluate the role of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in neuronal injury in experimental group B streptococcal meningitis, infected neonatal rats were treated with a monoclonal antibody against TNF- $\alpha$  (20 mg/kg intraperitoneally) or saline given at the time of infection. Histopathology after 24 h showed necrosis in the cortex and apoptosis in the hippocampal dentate gyrus. Treated animals had significantly less hippocampal injury than did controls ( $P < .001$ ) but had similar cortical injury and cerebrospinal fluid (CSF) inflammation. The antibody was then administered directly intracisternally (170  $\mu$ g) to test whether higher CSF concentrations reduced inflammation or cortical injury. Again, hippocampal apoptosis was significantly reduced ( $P < .01$ ), while cortical injury and inflammation were not. Thus, TNF- $\alpha$  played a critical role in neuronal apoptosis in the hippocampus, while it was not essential for the development of inflammation and cortical injury in this model.

Bacterial meningitis continues to be an important clinical problem, and morbidity and mortality remain unacceptably high. The prognosis is particularly poor in neonates, as reflected by mortality rates of 20%–40% and severe long-term neurologic sequelae in as many as one-third of the survivors [1, 2]. Long-term morbidity caused by meningitis includes hearing loss, hydrocephalus, and sequelae associated with parenchymal damage, including learning disabilities, memory loss, cerebral palsy, and seizure disorders. The mechanisms that lead to neuronal injury in meningitis are under active investigation. We have established an infant rat model of neonatal meningitis due to group B streptococci (GBS) that is well-suited for examining factors that contribute to neuronal injury [3]. Two forms of neuronal injury can be distinguished in the model, cortical neuronal necrosis and apoptosis of neurons in the dentate gyrus of the hippocampus [4].

The importance of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in bacterial meningitis has been well-established. Cell wall components of meningeal pathogens induce the release of TNF- $\alpha$  into the cerebrospinal fluid (CSF), and the cytokine plays an important role in the subsequent generation of subarachnoid space inflammation [5, 6]. In patients with bacterial meningitis,

CSF levels of TNF- $\alpha$  are elevated [7, 8]. High levels of TNF- $\alpha$  are of diagnostic value for bacterial meningitis and correlate positively with morbidity and mortality [7, 9, 10]. Furthermore, TNF- $\alpha$  may be involved in causing brain damage in bacterial meningitis. Work in experimental models of meningitis showed that TNF- $\alpha$  induced enhanced blood-brain barrier permeability and brain edema [11, 12]. Whether TNF- $\alpha$  contributes directly to the development of neuronal injury is not known. In the present study, we examined the role of TNF- $\alpha$  on the development of neuronal injury in the infant rat model of GBS meningitis, by use of a monoclonal antibody against TNF- $\alpha$  (TNF MAb). Treatment with antibodies against TNF- $\alpha$  has previously been shown to reduce blood-brain barrier permeability in hematogenous *Haemophilus influenzae* type b meningitis and to enhance survival in gram-negative sepsis and in GBS infection [13–15].

## Materials and Methods

**Infecting organism.** A GBS type III isolate, one of the most common types causing neonatal meningitis (gift of C. Rubens, University of Washington, Seattle), was used as described [3]. The organism was grown on blood agar plates, cultured overnight in 10 mL of Todd-Hewitt broth, diluted in fresh medium, and grown for 3 h to logarithmic phase. The suspension was centrifuged for 10 min at 5000 g and resuspended in normal saline to the desired density, and the absorbance was measured. An average inoculum size of  $10^4$  cfu was used to infect the rats.

**Model of meningitis.** Nursing Sprague-Dawley rat pups with their dams were purchased and infected on postnatal day 11 by direct intracisternal injection of 10  $\mu$ L of a suspension of the infecting organism with a 32-gauge needle. Control animals received an identical injection of sterile saline. Treatment with murine TNF MAb (Bayer, Berkeley, CA) or saline was started at the time of infection. In a first set of experiments, animals randomly received an intraperitoneal injection of 20 mg/kg TNF MAb ( $n = 21$ ) or the same amount of sterile saline ( $n = 19$ ). In a second set

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of experiments, 170  $\mu\text{g}$  (40  $\mu\text{L}$ ) of the TNF MAb ( $n = 19$ ) or an equal amount of saline ( $n = 17$ ) was directly administered intracisternally 30 min after infection. Pups were returned to their mothers, and 18 h later, they were weighed and assessed clinically for their ability to ambulate and right themselves. The presence of spontaneously occurring seizures was documented during the following 2 h. A sample of CSF (10–20  $\mu\text{L}$ ) was obtained by puncture of the cisterna magna. Bacterial titers in CSF were determined by plating 10-fold dilutions on blood agar plates and incubating the plates overnight at 37°C in room air. CSF lactate and glucose concentrations were determined by use of a two-channel autoanalyzer (YSI model 2300 G/L; Yellow Springs Instrument, Yellow Springs, OH) [16].

Animals were then treated with a single dose of ceftriaxone, 100 mg/kg subcutaneously. Twenty-four hours after infection, animals were sacrificed by intraperitoneal injection of pentobarbital (200 mg/kg). If an animal had to be sacrificed prematurely for ethical reasons or died spontaneously before 24 h, a randomly chosen littermate from the comparison group was sacrificed to assure similar survival times in the experimental groups (intraperitoneal experiments:  $23.5 \pm 1.4$  h in active group vs.  $22.5 \pm 1.9$  h in controls [not significant]; intracisternal experiments:  $22.4 \pm 2.0$  h in both groups). Animals that died unobserved were excluded from the analysis.

**Brain preparation.** Immediately after sacrifice, brains were removed and postfixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4°C and then placed in 30% phosphate-buffered sucrose. Brains were cut at 40–60  $\mu\text{m}$  thickness on a vibratome, and sections were mounted on gelatinized glass slides for staining. After dehydration, sections were stained by Nissl's method with cresyl violet, slides were rehydrated, and coverslips were fixed with Permount.

**End points.** Animals were weighed at 0 and 18 h after infection to assess weight loss. The degree of inflammation in the subarachnoid space was scored by a predetermined scheme: 0 = no inflammation; 1 = occasional inflammatory cells; 2 = inflammatory cells forming an infiltrate not involving the entire depth of the subarachnoid space; 3 = inflammatory infiltrate involving the entire subarachnoid space. Neuronal injury was scored for the cortex and the hippocampus. Cortical neuronal injury was defined as areas of decreased density of neurons or frank cortical necrosis and was quantitated as previously described [4, 17]. Briefly, 12 cortical sections spanning the middle half of the brain were scored in each animal for the presence or absence of neuronal injury, and the average injury score was used for statistical analysis. The scoring

system for cortical injury had previously been validated in a random set of animals ( $n = 8$ ) by computer-assisted quantitation of injury performed by an independent investigator. The correlation between the two scoring systems was excellent ( $r = .98$ ,  $P < .001$ ). Cortical injury in the present study was scored by a single investigator (M.G.T.) blinded to all experimental data.

Injured neurons in the dentate granule cell layer of the hippocampus were defined as cells showing markedly shrunken, condensed nuclei [4, 18]. Brain sections containing all four blades of the dentate gyrus were scored. Each blade was scored separately as follows: 0 = no injury; 1 =  $\leq 25\%$  of neurons abnormal; 2 = 25%–75% of neurons abnormal; 3 =  $> 75\%$  of neurons abnormal. Scores for all four blades were added and averaged for all sections. Injury in the hippocampus was scored independently by two investigators (M.G.T. and M.B.) blinded to the clinical, microbiologic, and treatment data of the animals, and the two scores were averaged for data analysis and presentation. The two scores of the two investigators correlated well ( $r = .71$ ;  $P < .001$ ).

**Statistics.** Weight loss and bacterial titers are presented as mean  $\pm$  SD, and differences between groups were analyzed by Student's  $t$  tests. Scores between groups were compared by the Mann-Whitney rank sum test. Seizure incidence and spontaneous death rates were compared by  $\chi^2$ .

## Results

### Systemic Administration of TNF MAb

**Clinical disease.** By 18 h after infection, all animals had meningitis, as evidenced by lethargy to obtundation, significantly altered CSF concentrations of lactate and glucose, and positive CSF bacterial titers (table 1). Treatment with TNF MAb had no significant effect on CSF bacterial titers and CSF concentrations of lactate and glucose (table 1). As a result of infection, all animals lost weight, but the weight loss was more pronounced in untreated animals than in animals treated with TNF MAb ( $3.0 \pm 0.5$  g vs.  $2.5 \pm 0.5$  g;  $P < .005$ ). Treatment with TNF MAb also significantly reduced the incidence of spontaneous death (24% in treated animals vs. 68% in controls;  $P < .02$ ).

**Histopathology.** Histopathologically, the disease in this model was characterized by an intense granulocytic inflammation in the subarachnoid and ventricular space and by two

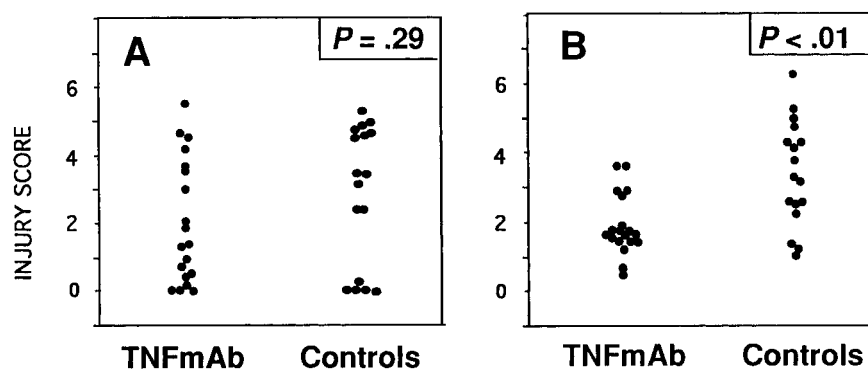
**Table 1.** Cerebrospinal fluid parameters in infant rats with experimental group B meningitis.

Parameter	Uninfected	Infected (at 18 h)		
		Controls	TNF MAb ip	TNF MAb ic
Bacterial titers (log <sub>10</sub> cfu/mL)	NA	$9.6 \pm 1.1$ (36)	$9.1 \pm 1.4$ (21)	$9.5 \pm 1.4$ (19)
Lactate (mEq/L)	$1.5 \pm 0.9^*$ (16)	$6.8 \pm 1.9$ (8)	$7.0 \pm 2.0$ (7)	$7.0 \pm 2.0$ (10)
Glucose (mg/dL)	$36.9 \pm 12.4^*$ (16)	$4.9 \pm 6.6$ (8)	$5.1 \pm 4.1$ (7)	$1.2 \pm 2.5$ (10)

NOTE. Data are mean  $\pm$  SD ( $n$ ). TNF MAb, monoclonal antibody to tumor necrosis factor- $\alpha$ ; ip, intraperitoneally; ic, intracisternally. NA, not applicable.

\*  $p < .001$  vs. all infected groups.





**Figure 3.** Injury scores in cortex (A) and hippocampus (B) of infant rats with group B streptococcal meningitis treated intracisternally with monoclonal antibody (MAb) against TNF- $\alpha$  or saline (controls). TNF MAb (170  $\mu$ g) or equal volume of saline was administered intracisternally 30 min after initiating infection. Statistics by Mann-Whitney rank sum test.

cally relevant concentrations, and a recent study using a human neuroblastoma cell line differentiated to a neuronal phenotype with retinoid acid showed that TNF- $\alpha$  induced apoptosis [21, 22]. There is also good evidence for a toxic effect of TNF- $\alpha$  in oligodendrocytes, the cell population responsible for myelin production in the central nervous system [23]. However, not all studies have found that TNF- $\alpha$  is involved in the development of neurotoxicity. In primary cultures of rat neurons, pretreatment with TNF- $\alpha$  promoted intracellular calcium homeostasis and protected neurons from damage caused by excitatory amino acids or hypoglycemia [24]. Similarly, a study in mice lacking TNF- $\alpha$  receptors found that ischemic and excitotoxic brain injury were increased compared with that in genetically normal control animals, indicating that TNF- $\alpha$  may have protective properties in this model of acute brain injury [25]. Thus, the role of TNF- $\alpha$  in acute neuronal injury is complex and likely influenced by specifics of the experimental system studied. In our rat model of neonatal meningitis, TNF- $\alpha$  appeared to be predominantly harmful, since several experimental end points, including neuronal apoptosis in the dentate gyrus of the hippocampus, were beneficially affected by treatment with TNF- $\alpha$  antibody.

The exact mechanisms by which TNF- $\alpha$  induced hippocampal apoptosis remain to be defined. A wealth of studies have documented that the cytotoxic effects of TNF- $\alpha$  are related to the production of oxidative radicals [26]. This pathway appears to be particularly critical in TNF- $\alpha$ -induced apoptosis, including in neuronal cells [22]. We have previously found in the infant rat model of meningitis that scavenging of reactive oxygen intermediates with the spin trapping agent  $\alpha$ -phenyl-tert-butyl nitron (PBN) was highly protective against apoptotic injury in the hippocampus, suggesting that oxygen intermediates play an important role in this form of injury *in vivo* [4]. Other mechanisms, however, could account for the effect of TNF- $\alpha$ . Cytokines may affect excitotoxic neuronal injury caused by glutamate and other excitatory amino acids, either by increasing their release from glial cells or by modulating excitatory amino acid receptors [21]. There is increasing evidence that excitatory amino acids can induce apoptosis in neurons under certain circumstances [27], and we have shown that they contribute to neuronal injury in the hippocampus in the

infant rat model of meningitis [17]. Thus, TNF- $\alpha$  could exert its effect by modulating excitatory amino acid-induced injury. Interestingly, excitatory amino acid receptor stimulation also leads to induction of reactive oxygen intermediates as important mediators of cellular injury [28]. Thus, regardless of whether TNF- $\alpha$  induces apoptosis in the hippocampus by a direct action on neurons or through modulation of the excitatory amino acid pathway, scavenging of oxygen radicals can be expected to be highly protective, as was the case in our study with PBN [4].

TNF- $\alpha$  did not appear to play an essential role in mediating subarachnoid space inflammation or cortical neuronal injury in the present study. These findings were surprising in light of previous studies in similar models of bacterial meningitis, in which TNF- $\alpha$  appeared to play an important role in generating CSF inflammation and in which antibodies to TNF- $\alpha$  were effective in reducing CSF inflammation [5, 11]. Our results are likely not a reflection of a general lack of biologic activity of the TNF MAb used in the study, since the antibody showed significant beneficial effects on hippocampal injury as well as systemic parameters, seizure activity, and mortality. Furthermore, experiments in which the TNF MAb was administered intrathecally indicated that its limited effect was not likely the result of inadequate CSF concentrations. In these studies, ~20% of the systemic dose of the antibody was injected directly into the CSF, resulting in estimated CSF concentrations 10- to 100-fold higher than after systemic administration. An important difference between the present study and previous studies that may explain the discrepancy is the time points of examination. Previous studies focused on early time points up to 6 h after infection, while animals were examined 18–24 h after infection in the present study. It is conceivable that TNF- $\alpha$  is critical early in infection, while other inflammatory mediators may compensate for TNF- $\alpha$  in later stages of the disease.

The differential effect of TNF MAb on hippocampal and cortical injury underlines the possibility that different mechanisms may be involved in these two forms of injury. Previous studies indicated that cortical injury is caused primarily by ischemia, which in turn is likely to be a result of the effect of subarachnoid space inflammation on the cerebral vasculature [4, 17]. Given this causal relationship, it is not surprising that

the failure of TNF MAb to affect CSF inflammation resulted in a corresponding lack of protection against ischemic cortical injury. Hippocampal injury, on the other hand, does not appear to be of ischemic origin, since the ischemia-sensitive structures of the hippocampus (CA1/CA3) are not affected in the meningitis model and since the dentate gyrus is not considered to be particularly ischemia-sensitive. It appears more likely that soluble mediators, such as TNF- $\alpha$  or excitatory amino acids, have direct effects on the neurons in the dentate gyrus, possibly by diffusing from the ventricular space, which is in close proximity to the dentate gyrus and shows uniform inflammation in the model [17].

The clinical significance of the hippocampal injury observed in our model, and thus of the selective beneficial effect of TNF MAb on this form of neuronal injury, needs to be further explored. Neonatal meningitis often results in mental retardation and learning disabilities [2], and the selective injury to the hippocampus in our model is intriguing, since this structure plays a critical role in learning and memory functions. It is unknown whether similar changes can be observed in humans after meningitis, but similar findings in pneumococcal meningitis in rabbits indicate that hippocampal injury is not unique to our infant rat model [18]. Independent of these questions, our study significantly expands the understanding of the molecular mechanisms of brain injury in meningitis by documenting a critical role of TNF- $\alpha$  in causing apoptotic cell death in a selected subpopulation of neurons.

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